Editorial Review

Monitoring of lymphocyte subpopulation changes in the assessment of HIV infection

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If the effects of the human immunodeficiency virus (HIV) on the central nervous system are discounted, the majority of the major clinical manifestations of HIV infection can be explained by the selective and progressive depletion of the CD4 bearing T lymphocyte subpopulation following the infection of this cell type by HIV. Physiologically, the CD4 T lymphocyte is the cell type first involved in the recognition of antigen presented by Class II major histocompatibility complex bearing antigen-presenting cells. It serves as the pivotal cell in amplification of immune responses through the release of cytokine mediators, principally interleukin 2, interleukin 4 and gamma interferon. Following antigen recognition by CD4 cells, released gamma interferon is an important activator of macrophage/monocytes which play a key role in the destruction of intracellular infections of macrophages such as the mycobacteria, toxoplasma, salmonella and various parasitic species which typify the later stages of HIV-associated immunodeficiency. Since in both blood and lymphoid organs the degree of CD4 depletion in the terminal stages of HIV infection is as severe as that encountered in major congenital T cell deficiency states such as the di George Syndrome or severe combined immunodeficiency, it is to be expected that the degree of clinical immunodeficiency is wide ranging and severe.

The majority of cohort studies,[1–5] have with few exceptions,[6] documented that HIV produces slow and predictable attrition of the CD4 population from the time of seroconversion. It has become increasingly evident that absolute CD4 counts are predictors of progression to AIDS and operate independently of other measurements such as serum p24 antigenaemia or raised serum beta 2 microglobulin.[3 7] These prospective studies indicate that over a three year period of follow up few HIV individuals with presentation CD4 counts above 500/mm^3 progress to AIDS whereas the majority of individuals with initial counts below 200/mm^3 will do so.

Although other abnormalities of lymphocyte populations have been consistently noted in HIV infected patients most particularly the elevation of CD8 T cells and the expansion of Class II bearing T cell percentages, the association with clinical progression of these other changes has not been extensively investigated in comparison to information available for CD4. More recently it has been suggested that in individual patients the level of CD4 cells may provide the basis for clinical decision making. The most impressive evidence has been provided from data presented in a National Institute of Health study in which it was clearly documented that *Pneumocystis carinii*, cytomegalovirus or atypical mycobacteria species were virtually never demonstrable as causes of pulmonary disease in HIV infected individuals who had CD4 counts of greater than 200/mm^3 or 20% of total lymphocytes in the 60 days prior to the development of pneumonitis.[8] This study has been recently confirmed by another group with very similar findings.[9] It is on the basis of this evidence that the Centres for Disease Control advocated the introduction of primary prophylaxis against pneumocystis in all patients with CD4 counts below 200 mm^3.[10] Advance information made available from the as yet unpublished 014 zidovudine/placebo trial of the National Institute of Allergy and Infectious Diseases also claims that zidovudine delays progression in asymptomatic HIV infected subjects with CD4 counts below 500/mm^3. The consequence of these reports has been to heighten interest in the use of CD4 counts in individual patients to assess risk of progression and response to therapy.

However, if lymphocyte marker counts are to be used discriminatingly and successfully in such a role it is vital that individual physicians understand the basis for the interpretation of these results and the methodological and clinical pitfalls that can produce the fluctuating or erroneous values which have led some to question their value.

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THE TECHNOLOGY EMPLOYED IN LYMPHOCYTE PHENOTYPING

Until recently lymphocyte surface marker analysis was principally used as a research procedure. Early analysis techniques used monoclonal antibodies to antigens expressed by individual lymphocyte populations to identify specific markers on whole mononuclear cell preparations separated from blood. Individual cells were usually identified by immunofluorescence microscopy after addition of a second layer fluorescent anti-immunoglobulin. These techniques had large intrinsic margins of error. Lymphocyte separation techniques may lead to selective loss of individual cell populations and double layer fluorescent techniques were prone to problems of non-specific antibody binding, particularly to monocytes. However, the greatest single variable lay in the visual microscopic assessment of a limited number (usually only 100–200) of cells. For statistical reasons alone the confidence limits of such techniques were unacceptably wide. The accuracy and precision were further compounded if the observer was inexperienced in distinguishing individual cell types or fluorescent thresholds.

Until recently these considerations were not of major importance. Prior to the assessment of HIV infection the only areas of clinical medicine in which immunofluorescence phenotyping was routinely applied in diagnosis were in leukaemia/lymphoma typing and in the assessment of major congenital immunodeficiencies. In both these disease groups the question being asked was whether a monoclonal or total cell population was present in blood or not, and therefore, the assessment was essentially qualitative rather than quantitative. Thus interassay precision and accuracy of individual counts were relatively unimportant to laboratories involved in performing early lymphocyte phenotypic analysis.

The introduction of flow cytometers allowed the rapid quantitation of very much larger numbers of lymphocytes (usually 3,000–10,000). It greatly increased precision and accuracy of these investigations as well as removing the subjectivity inherent in fluorescence microscopy. More recently the introduction of directly conjugated monoclonal antibodies which reduce the problems of non-specific staining and more importantly allow the use of whole-blood fluorescence and double marker techniques have removed the potential for selective loss of cells at the lymphocyte separation stage. When AIDS became prevalent in the early 1980s most teaching centres already had access to flow cytometry (FACS) facilities. However, following the identification of HIV and the introduction of laboratory safety guidelines many of these research instruments were not made available for analysis of HIV infected material. As a result microscopy with its inherent imprecision continued in widespread use. More recently second generation flow cytometers have been introduced. They are simpler to operate, offer improved operator safety and are less expensive to purchase. They have increased the availability of the flow cytometry so that it is now available for HIV investigation in most larger centres. However, until now flow cytometry has remained a semiquantitative technique. Attention has only recently been directed to the possible reasons for variability in individual results.

TRENDS IN CD4 COUNT DURING THE NATURAL HISTORY OF HIV INFECTION

In the early asymptomatic phase of HIV infection, CD4 counts may remain stable and within quoted adult normal ranges for long periods of time. A small and diminishing proportion of adults show normal CD4 counts five years or more after documented HIV infection. Most large published cohorts however indicate that CD4 counts fall progressively in most infected patients and this trend can be established in virtually all patients by sequential monitoring.[2 4] Indeed, it is the consistency of this decline in all studied cohorts that has resulted in the predictions for the total attack rate for HIV infection being regularly updated towards percentages approaching 100%. The rate of fall of CD4 cells is faster in some patients than in others. The reasons for this variation are only partially understood. Age is a clear influence with older[11] and very young patients progressing more rapidly. One recent study suggests that patients with prolonged acute seroconversion illnesses also display accelerated progression rates.[12]

Some studies suggest that the rate of CD4 decline accelerates in the months before a patient progression to AIDS[2 13] but not all reports confirm this impression.[4] In some patients the first major opportunistic infection appears to result in a final abrupt loss of CD4 cells which fails to return to premorbid levels after successful treatment of the index infection. However, lymphocyte surface marker results obtained during a period of acute infection may be misleading. Acute sepsis has been documented to produce marked transient changes in lymphocyte subpopulations in the absence of HIV infection[14] and in our experience acute bacterial sepsis will produce similar temporary changes in HIV infected subjects. It is important, therefore, that if CD4 counts are to be used to stage patients clinically they must *not* be taken during an acute exacerbation of disease or during an irrelevant intercurrent infection. This consideration emphasises the need for clinical follow-up and baseline monitoring during the long asymptomatic phase of HIV infection.

A further cause of clinical variability is diurnal variation in lymphocyte counts. Total white cell counts and the derived lymphocyte subset values are subject to predictable and marked variation.[15] Peak

CD4 counts in normal individuals are found at 2300 h and trough levels at 1100 h. Counts are inversely correlated with plasma cortisol levels. The effects are substantial with counts varying by up to 50% between 1600 and 1700 h. Limited studies suggest that in HIV infected subjects with normal or moderately reduced CD4 counts such variations can also be demonstrated and can result in apparent but spurious immunological deterioration if sequential samples are taken during clinic attendances at different times of day.

Clinicians should be aware of this substantial source of individual variation and either attempt to limit its effects by synchronising clinic visits or to discount for its possible effects in assessing results from discordant timing of visits. To further complicate the picture, two groups have described variation by time of year (circannual) amongst normal volunteer donors.[16 17] Circannual variation produces both accentuation or flattening of individual diurnal variation depending on the time of year, as well as significant changes in absolute values from month to month at the same time of day. In one study the mean CD4 count rose by 40% from June to November in one individual in a pattern that was reproducible year on year.[17]

In addition to these procedural variables in the time of collection of samples, and condition of the patients, certain aspects of laboratory practice may also influence lymphocyte phenotype results. Delay in analysis of samples and fluctuations in temperature of samples during transit may affect results.[18 19] Recent studies suggest that provided that samples are prepared within 24 hours of venesection and transported in anticoagulant at an ambient temperature of 22°C, then lymphocyte subpopulation results are relatively stable.[20] Deviation from this protocol will significantly affect results of subpopulations of lymphocytes but delay in analysis of more than 6 hours have much more marked effects on total white cell counts performed by automated haematology counters. Beyond 6 hours automated white cell counters will begin to reject samples because of cell degeneration.[21] This may be one explanation why in many hands subpopulation percentages are more concordant than absolute numbers on sequential monitoring in HIV infected subjects.[22]

SUBPOPULATION PERCENTAGES, ABSOLUTE COUNTS AND RATIOS

Careful studies on normal volunteers have demonstrated that the stability of lymphocyte phenotype counts as expressed by coefficients of variation on percentage counts, vary little on the same individual (4–10%) but vary much more widely between different individuals. This stability is reassuring in the context of monitoring HIV infected patients, since each individual can serve as their own control in assessing trends. However, since these studies were performed on laboratory volunteers under optimal conditions (same time of day, rapid analysis time etc) they probably represent an ideal which can only be partially attained in the clinical situation.

Most studies agree that in the follow-up of HIV infected patients the percentages of CD4 counts and to a lesser extent CD8 counts are relatively consistent over short time periods and begin to show identifiable trends in the majority of patients over longer intervals. Most studies of quality assessment and biological variation have concentrated on percentages of lymphoid populations. However, it is generally accepted that conversion of percentage counts to absolute values derived from the total lymphocyte count is more immunologically relevant. This is justified by the knowledge that percentages are relative and in the case of the mutually exclusive CD4 and CD8 markers can be influenced by a fall in one population or a rise in the other. For example, in acute infectious mononucleosis the CD4 percentage is low and the 4/8 ratio markedly reversed. This is not because CD4 populations are reduced, but rather because primary Epstein-Barr virus infection is characterised by an acute rise in total CD8 lymphocytic population, which largely comprises the "atypical mononuclear" cells that typify that disease. Thus, lymphocyte percentages interpreted in isolation may be misleading. However, a number of studies and general exeperience suggests that in the longitudinal assessment of HIV infected patients, results expressed as percentage CD4 results often give a more stable picture and clearer indication of trends than do absolute values.[22]

The reason for the greater apparent stability of percentages is because the absolute CD4 count is derived from multiple variables, the CD4% and the absolute lymphocyte count. The absolute lymphocyte count itself contains two variables, the enumeration of the total white cell count and also the differential performed on that count to identify its component cell populations. Since the total white cell count itself is labile, being affected by factors which include diurnal influence, exertion, intercurrent infection etc, it is to be expected that in general the absolute lymphocyte count tends to vary more widely than do the proportions of its lymphocyte subpopulation components.

A further source of error results from two specific laboratory practices which are particularly relevant to the investigation of HIV infected patients. The first is that many laboratories batch "high risk" samples and run them at the end of the working day, or at the beginning of the next. As already discussed, numerous studies have shown that delay in analysis produces considerable variations in the quantitation of absolute white cell counts. Whilst this may be

relatively unimportant in many clinical situations in which the white cell count is used only as a semiquantitative guide, it matters greatly when precision counting is required to assess lymphocyte subpopulation trends in individual HIV infected patients. Secondly, some laboratories perform differential counts on "high risk" and indeed on normal subjects using a microscopically assessed visual differential in which only 100–200 cells are counted. As already discussed, the wide confidence limits inherent in such small count samples, renders such procedures intrinsically imprecise. This consideration is often linked to the first, if analysis is delayed automated counters often reject the aged samples and laboratories then resort to manual techniques to obtain a result. Such considerations probably explain why the absolute white cell counts perform generally less well than the percentage T lymphocyte subpopulations in quality assurance exercises (MRC/Inserm Concorde UK quality control returns—unpublished data). When combined with the biological variation already discussed, it is not surprising that these individual considerations summate to produce a significant degree of fluctuation between individual CD4 absolute values. Nevertheless, it is the absolute number of CD4 cells which largely determines short and medium term clinical outcome. This consideration combined with the potential errors of interpretation that can result from the use of percentages or ratios suggests that absolute values should be used in preference. Clinicians and laboratories should pay attention to all aspects of potential biological and procedural variation with the aim of improving the precision and accuracy of lymphocyte subpopulation percentages and the derived absolute counts.

CONCLUSIONS

Although there is general agreement that CD4 measurements provide reliable prognostic information within cohorts of HIV infected subjects, results in individual patients exhibit fluctuations which require care in interpretation. The fluctuation may contain two elements, biological variability within each individual and imprecision due to intrinsic errors of measurement. Biological variation can be minimised, but not eliminated by adopting a standard protocol within and between centres for the withdrawal and dispatch of samples, by attention to diurnal influences and by excluding the effects of acute infections, intercurrent illness or relevant drug therapy. Measurement errors can be minimised by the adoption of standardised laboratory procedures for both lymphocyte phenotypic analysis and in the automated enumeration of absolute white cell and differential counts. The introduction of national and international laboratory quality assurance schemes which address these factors should facilitate rapid progress towards standardisation and minimisation of measurement errors.

A further strategy to reduce the degree of count fluctuation is to obtain more frequent sample analysis so that individual trends can be more precisely assessed and outlying results rejected. If future trials of candidate anti-retroviral drugs or immunomodulatory agents are to include laboratory measurements such as CD4 trends as indicators of therapeutic efficacy then it is probable that lymphocyte subpopulation analysis will be required more frequently than is currently being employed in the routine clinical follow-up of asymptomatic HIV infected patients.

Most important of all is the need for clinicians involved in the care of HIV infected patients to establish and maintain a dialogue with the immunology and haematology laboratories responsible for providing their laboratory data. Clinicians should remember that quality assurance begins at the time of specimen collection and ends with the dispatch of the result from the laboratory. Attention to detail at all stages will result in a steady improvement in the quality of the laboratory data obtained, and should extend the design and power of future trials directed at improved therapeutic approaches towards HIV disease.

Table Summary of factors influencing the stability of lymphocyte subpopulation counts

BIOLOGICAL
Acute intercurrent infection
Drug therapy
Diurnal variation
Stress and exertion
PROCEDURAL
Temperature of specimen in transit
Delay in analysis
Changes in laboratory analytes (antibodies)
Changes in laboratory procedure/methodology

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Correction

On the cover of *Genitourin Med* April 1990 the paper "The changing pattern of antibiotic resistance of *Neisseria gonorrhoeae*" was wrongly attributed, and was by Charles S F Easmon.